

The mechanical properties of amniotic membrane influence its effect as a biomaterial for ocular surface repair

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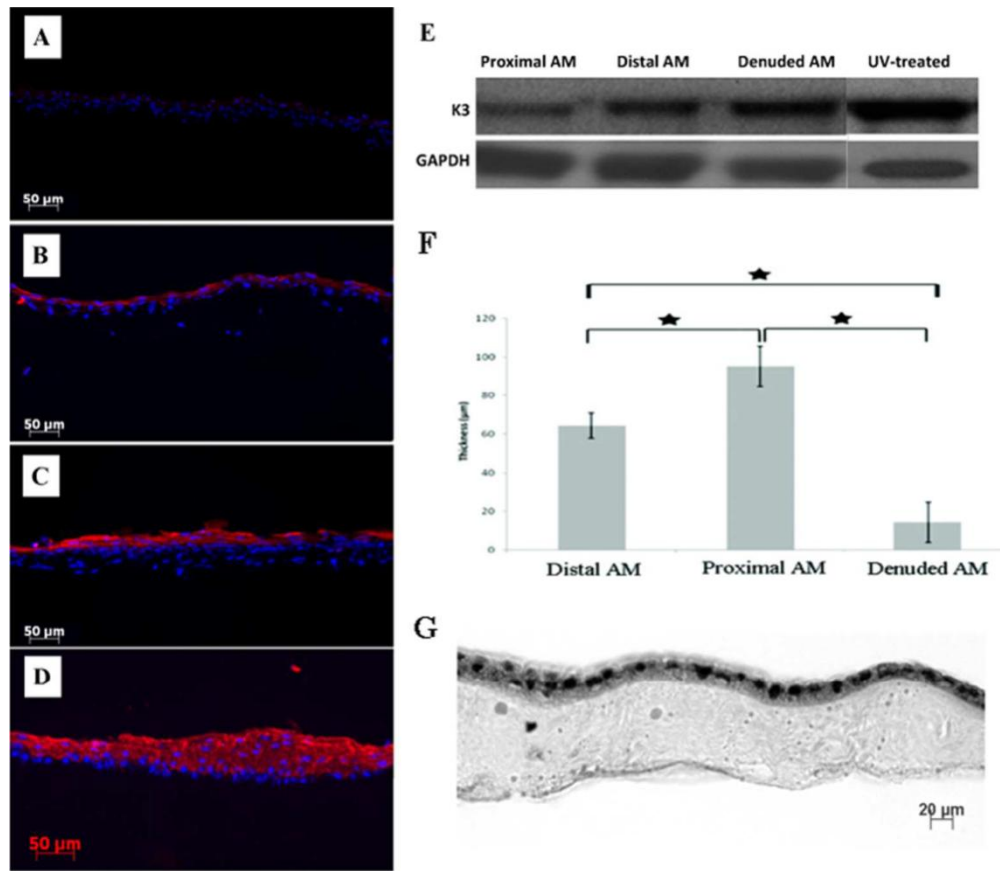
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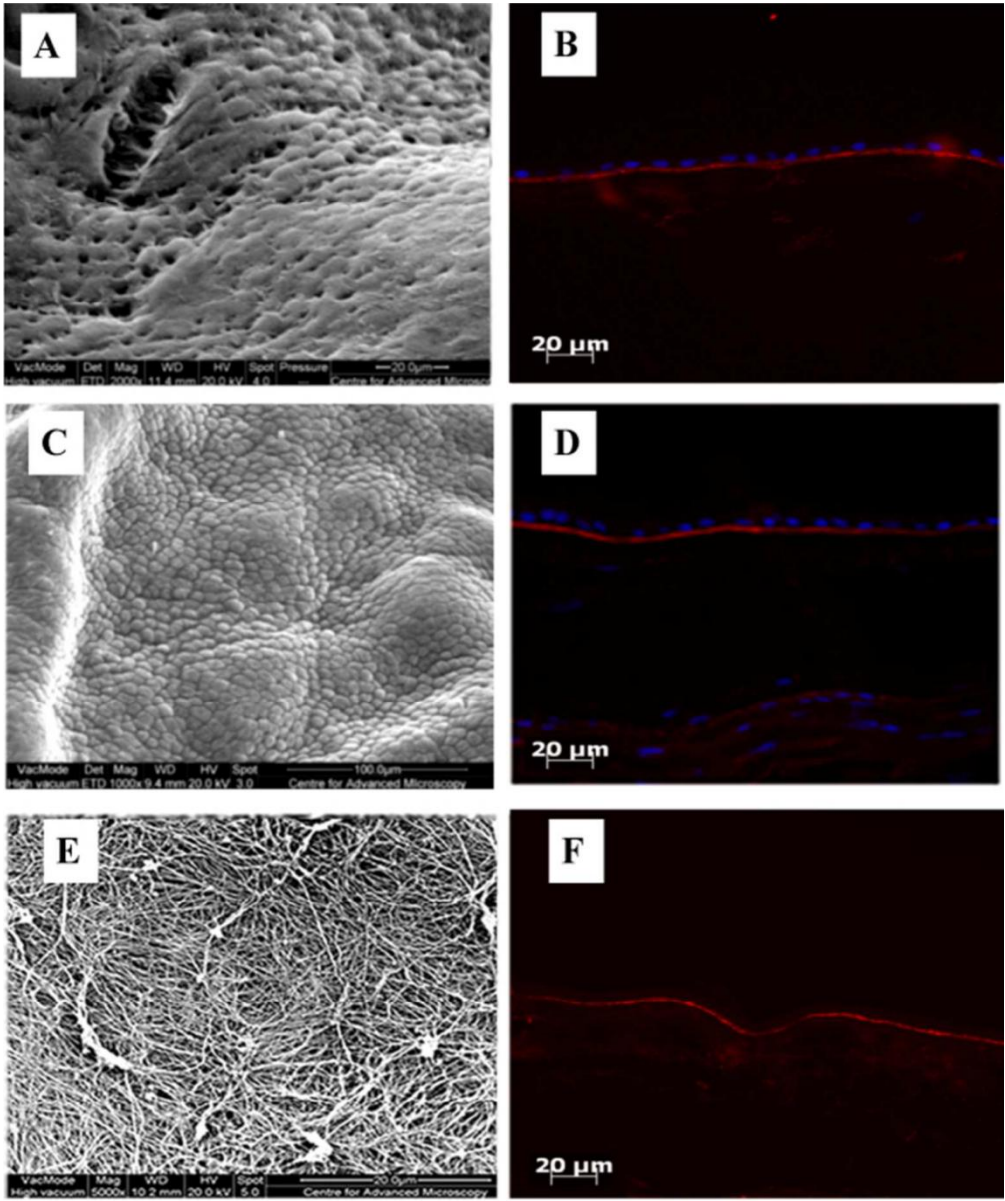
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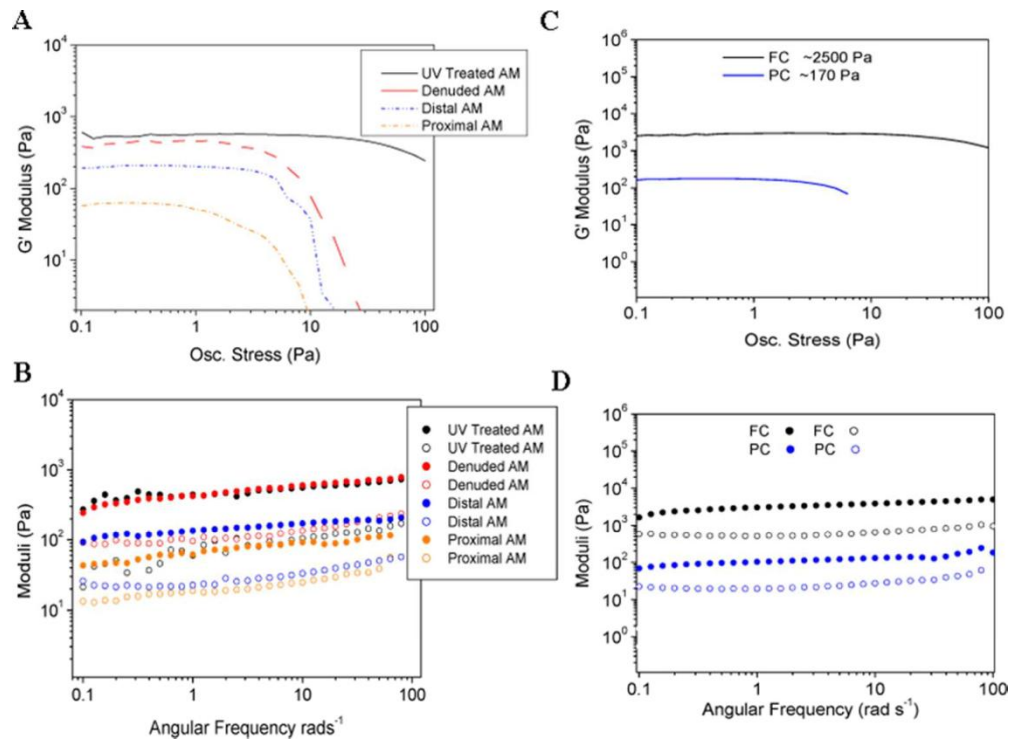
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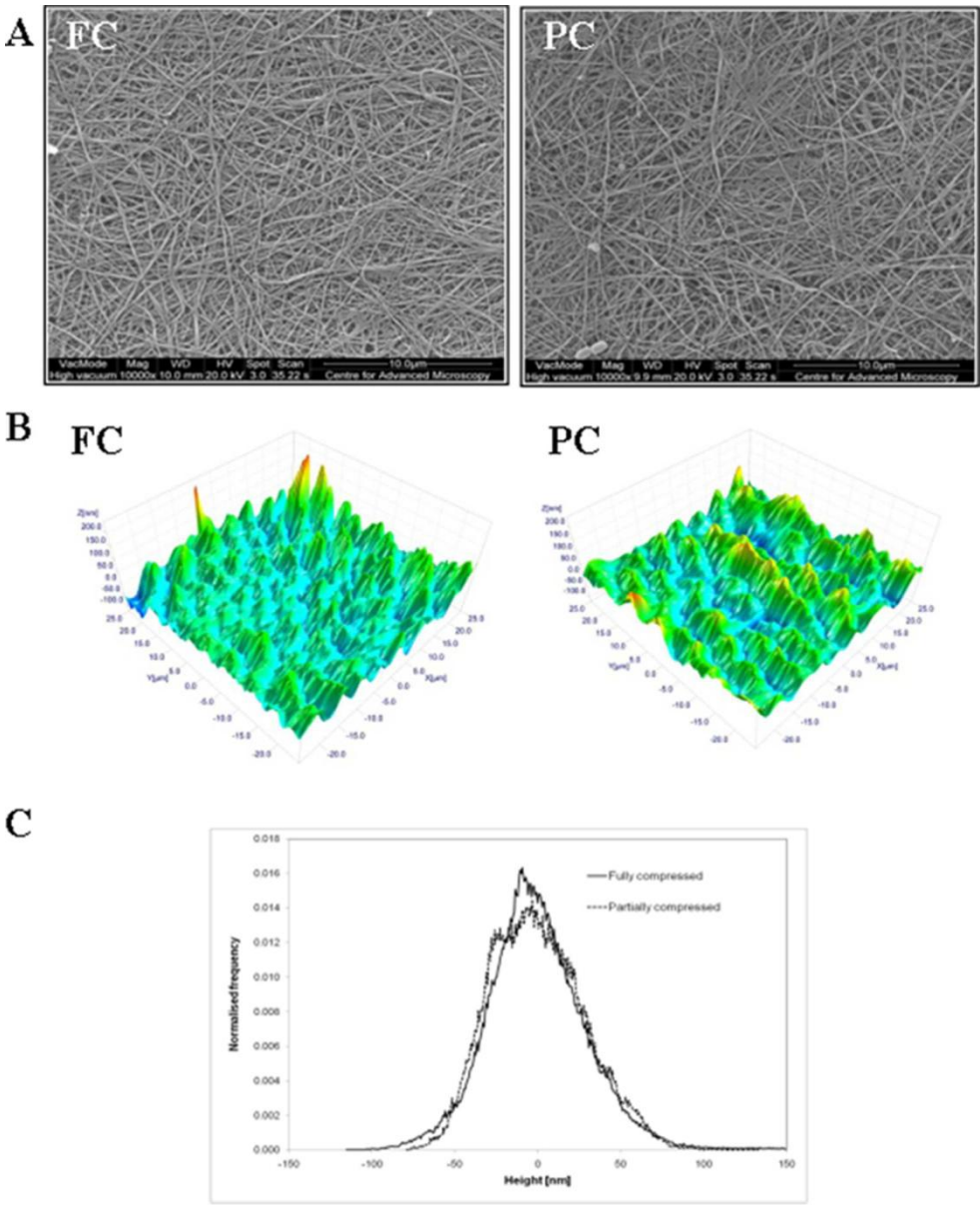
K3 expression (red) in limbal epithelial cells cultured on (A) Proximal (B) Distal (C) Denuded and (D) UV cross-linked amniotic membrane. Cell nuclei stained by DAPI (blue). K3 expression is seen to increase its association with deeper layers of stratified cells from proximal>distal>denuded>UV treated amniotic membrane. This increase was also apparent by Western blotting against K3 with GAPDH as a loading control (E). The thickness of amniotic membrane was measured ($n \geq 9$) and a significant difference in thickness was found between distal, proximal and denuded AM (* = $P < 0.01$). Error bars equal standard deviation of the mean (F). A representative image showing the thickness measurement of distal amniotic membrane (G) 63x54mm (300 x 300 DPI)



The ultrastructure and collagen IV expression (red) upon the surface of distal (A, B), proximal (C, D) and denuded (E, F) amniotic membrane prior to its use in the expansion of limbal epithelial cells was examined by scanning electron microscopy and immunohistochemistry respectively. Amniotic epithelial cells appear more loosely attached in the distal tissue (A). The denuding process whilst removing the amniotic epithelia does retain a similar expression pattern of collagen IV across its upper surface (F).
55x65mm (300 x 300 DPI)

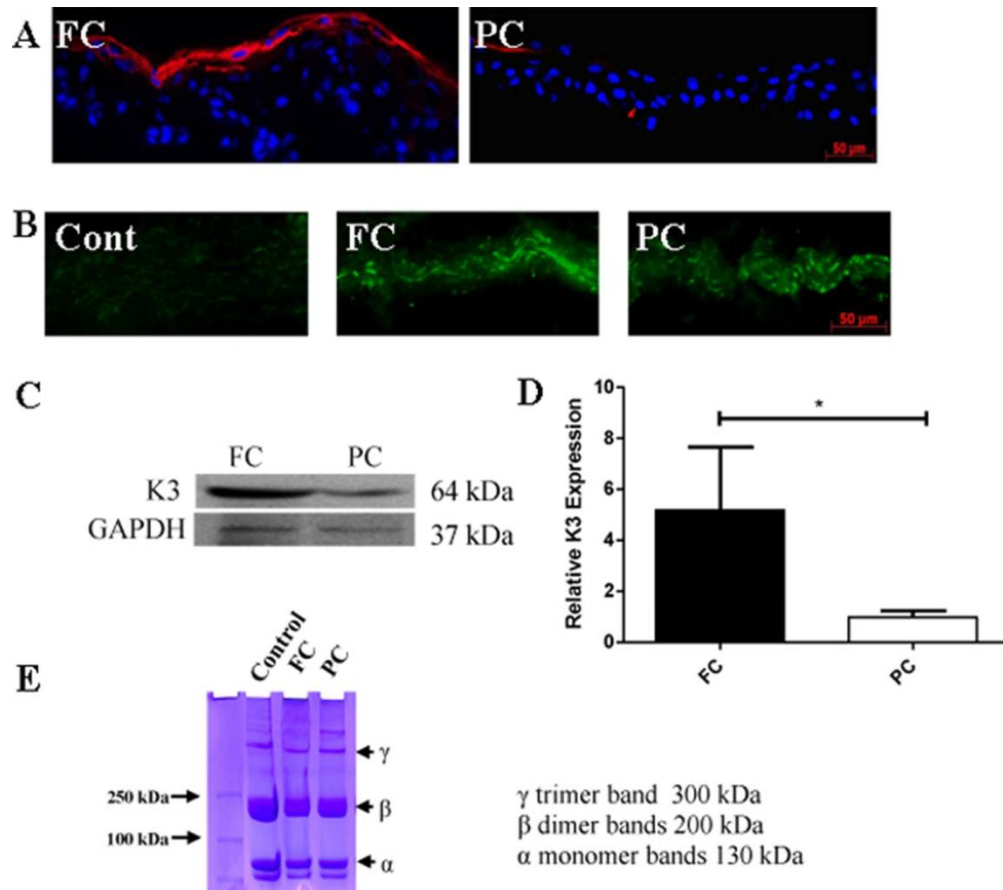


Rheology data showing the mechanical properties of proximal, distal, denuded and UV treated human amniotic membrane (A, B) and fully [FC] and partially compressed [PC] collagen gels (C, D). Oscillatory stress sweeps (A, C) and frequency sweeps (B, D) are shown for each substrate. Closed symbols refer to G' (dynamic elastic shear modulus), open symbols refer to G'' (dynamic viscous shear modulus).
70x51mm (300 x 300 DPI)



Scanning electron micrographs from the surface of fully compressed [FC] and partially compressed [PC] collagen gels reveal a similar ultrastructure in terms of collagen fibre density and diameter (A). Atomic force microscopy across 50µm × 50µm surface regions of the fully and partially compressed collagen gels revealed a similar surface topography by both 3D mapping (B) and a distribution of peak heights histogram (C).

60x73mm (300 x 300 DPI)



Immunohistochemical staining of limbal epithelial cells on fully compressed [FC] and partially compressed collagen [PC] gels (A). K3 (red) is predominately associated with superficial cells expanded upon the stiffer fully compressed collagen gel. Nuclei counterstained with DAPI (blue). Laminin distribution (green) was similar between the fully compressed and partially compressed collagen gels, negligible amounts were detected in the control gel without laminin coating (B). Western blotting results show an increased K3 (64 kDa) expression from epithelial cells expanded upon stiff collagen gels [FC] when compared to the less stiff [PC] collagen gels (C). This difference was also quantified by QPCR showing a significant increase in K3 mRNA levels when expanded upon the stiffer collagen gels (D). Coomassie blue staining of collagen fragments in fully compressed [FC] and partially [PC] collagen gels revealed no obvious difference in the relative amounts of type I collagen fragments (gamma, beta and alpha) (D). Control is an uncompressed collagen gel.

53x47mm (300 x 300 DPI)

Substrate	G' (Pa) at 1 rads ⁻¹
UV Treated AM	441.60 ± 27.27
Denuded AM	423.1 ± 89.28
AM proximal to placenta	135.27 ± 15.27
AM distal to placenta	61.68 ± 15.65

Dynamic elastic shear modulus measurements (G') of amniotic membrane revealed an increase in stiffness (UV/riboflavin>denuded>distal>proximal) with increasing sampling distance from the placenta and subsequent processing (removal of native epithelia and chemical cross-linking).
69x20mm (300 x 300 DPI)

The mechanical properties of amniotic membrane influence its effect as a biomaterial for ocular surface repair

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Abstract

The human amniotic membrane (AM) is a tissue of fetal origin and has proven to be clinically useful as a biomaterial in the management of various ocular surface disorders including corneal stem cell transplantation. However its success rate displays a degree of clinical unpredictability. We suggest that the measured variability in AM stiffness offers an explanation for the poor clinical reproducibility when used as a substrate for stem cell expansion and transplantation.

Corneal epithelial stem cells were expanded upon AM samples possessing different mechanical stiffness. To investigate further the importance of biological substrate stiffness on cell phenotype we replaced AM with type I collagen gels of known stiffness. Substrate stiffness was measured using shear rheometry and surface topography was characterized using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The differentiation status of epithelial cells was examined using RT-PCR, immunohistochemistry and Western blotting.

The level of corneal stem cell differentiation was increased in cells expanded upon AM with a high elastic modulus 423.30 Pa (± 89.28) and cell expansion on type I collagen gels confirmed that the level of corneal epithelial stem cell differentiation was related to the substrate's mechanical properties.

In this paper we provide evidence to show that the preparatory method of AM for clinical use can affect its mechanical properties and that these measured differences can influence the level of differentiation within expanded corneal epithelial stem cells.

1 Introduction

2 Severe ocular surface disorders¹⁻⁶ can cause significant morbidities and even corneal blindness.

3 For several decades in vivo and in vitro based evidence has proven amniotic membrane (AM) to
4 be an excellent natural substrate for the expansion of corneal specific (limbal) stem cells to aid in
5 ocular surface repair⁷⁻¹⁰. The success of AM has been in part due to the ease in which cultivated
6 corneal epithelial cells can form a stratified tissue whilst maintaining a high proportion of
7 undifferentiated stem cells¹¹⁻¹⁴, thus forming a tissue suitable for transplantation to a diseased or
8 damaged human ocular surface.

9
10 Despite the structural and biological properties of AM being poorly understood it has been
11 widely used in the study of corneal epithelial cell biology, as well as a popular adjunct for ocular
12 surface reconstruction^{7, 15, 16}. The long and continued debate on the best methods for AM
13 preparation, prior to its use as a substrate for ex vivo corneal epithelial cell expansion, is a case in
14 point^{12, 17, 18}. Furthermore, regardless of AM's many reported successful ophthalmic outcomes,
15 the resulting overall clinical efficacy has lacked predictability^{16, 19-21} its success rate, when used
16 in conjunction with stem cells, ranges from 46% to 100%^{15, 19, 22-28}. These variations have been
17 partially explained by differences in biochemical or topographical cues and/or storage conditions
18 prior to AM use²⁹.

19
20 Previously AM has been reported to contain a variety of soluble growth factors and cytokines³⁰⁻³²,
21 most of which are associated with various stem cell activities. Although the surface topography
22 of AM has been shown to influence the fate of stem cells¹⁴ the main factors underlying the cause
23 of clinical unpredictability have yet to be identified.

We have reported previously that various anatomical regions of AM have unique structural properties, specifically that there exists a loss of lattice-like arrangement in collagen organization from areas near to the normal site of AM rupture at term³³ as well as a loss of collagen density, material thickness and transparency³⁴. These findings suggest that intra and inter sample variation in AM structure might be the root cause of AM's varied clinical effectiveness.

Recently several studies have demonstrated the importance of substrate stiffness in the regulation of stem cell differentiation³⁵⁻³⁸. Therefore, we hypothesized that the known variances in AM structure may influence its mechanical properties and subsequently affect the level of corneal epithelial stem cell differentiation; and that these differences could explain the inconsistent clinical results when AM is used as a substrate for limbal stem cell expansion and transplantation.

Materials and Methods

Preparation of human amniotic membrane

Human fetal membrane was collected under sterile conditions from healthy donor pregnant women following delivery by elective term caesarean section at University College London Hospital (UCLH), United Kingdom (n>6). The tissue was processed as previously reported with modifications²³. Briefly, following delivery the membranes were washed by sterile phosphate-buffered saline (PBS) containing penicillin (100IU/mL) and streptomycin (100 mg/100mg/mL) (Life technologies, U.K.) within a class II microbiology safety cabinet. The chorion (a separate layer beneath the AM) was peeled away and discarded; the AM samples were taken from areas adjacent to the placental disc (proximal AM) and approximately 10 cm from the placental disc (distal AM). These samples were washed a further five times for 15 minutes each with PBS. Once thoroughly cleaned, the proximal and distal samples were cut into smaller pieces (4 cm), washed again in PBS and immediately stored in 50 ml PBS at -80°C.

Prior to use, the cryo-preserved AM samples were thawed and washed twice more in sterile PBS for 10 minutes and the remaining AM epithelial cells were mechanically removed (denuded). Denuding was achieved by incubating the AM samples in 0.1 % ethylenediaminetetraacetic acid (EDTA, Invitrogen) at 37 °C for 1 hour to lessen cellular adhesion, followed by vigorous rubbing with cell scrapers (Fisher Scientific, UK). Previously we and others have shown that this process removes both the native epithelia and basement membrane exposing the bare stroma underneath^{14, 63}.

1 Thickness measurements of the prepared amniotic membrane samples

2 AM thickness measurements were taken from 10 μ m transverse cryo-sections stained with
3 heamatoxylin and eosin (H&E) and recorded using a calibrated light microscope with an
4 attached digital camera (Axioskop 2, Zeiss, Germany). Three contiguous serial sections were
5 taken from three different lateral regions of both the proximal and distal AM samples . Ten
6 thickness measurements were taken from each section, and the results were averaged.

7
8 Photochemical cross-linking treatment of amniotic membrane

9 Photochemically cross-linked AM were formed by irradiating the denuded AM to UV light
10 similar to the ophthalmic procedure for the treatment of keratoconus⁵². In brief, denuded AM
11 was incubated in a saturated riboflavin solution in 20 % (w/v) dextran (Dextran T500, 500 KDa,
12 Sigma, U.K.) for 5 minutes immediately before irradiation. The irradiation was performed for 30
13 minutes using a UV lamp (UVP, B-100AP, U.K.) at 365 nm at distance of 110 mm from the
14 sample. The photochemically treated samples were then washed 3 times with phosphate buffered
15 saline (PBS, Fisherfisher, U.K.), then transferred directly into the base of 6 well tissue culture
16 plates (transwell, Costar, U.K.) ready to act as a substrate for limbal epithelial cell expansion⁵³.

17
18 Preparation of type I collagen gel (as a synthetic amniotic membrane)

19 The material structure of AM's extracellular matrix was mimicked using a compressed (stiffened)
20 type I collagen gel. Compressed collagen gels were prepared as previously described^{49, 61, 64} with
21 some modifications. Briefly, collagen gels were prepared by neutralizing 4 ml sterile rat-tail type
22 I collagen (2.2 mg/ml in 0.6 % acetic acid, First Link Ltd) in 1 ml modified Eagle's minimum
23 essential medium (Life technologies, U.K.) and 0.5 ml 1 M sodium hydroxide (Fisher, U.K.).

The solution was gently mixed and cast into rectangular moulds (33 mm x 22 mm x 8 mm) prior to gelling at 37 °C, 5 % CO₂ for 30 minutes. Partially compressed (PC) and fully compressed (FC) collagen gels were then prepared by variable compression between a layer of nylon mesh (50 µm mesh size). PC gels were generated by compression under a fixed load of 64 g for 2.5 minutes whilst FC gels were compressed under 134 g for 5 minutes both at room temperature⁶⁴.

Culture of bovine limbal epithelial cells

Fresh corneas were dissected from bovine eyeballs prior to epithelial cell isolation. Limbal tissue was cut into 10–12 pieces (5 mm long, 3 mm wide) and incubated for 12 hours at 37 °C in a basal culture medium with 0.02 % type IA collagenase (Sigma-Aldrich). The epithelium was peeled from enzyme treated limbal pieces, incubated with 0.05 % trypsin–ethylenediamine-tetraacetic acid (Sigma-Aldrich) for 10 minutes at 37 °C, and the cell sheets were dissociated into single cells by agitation through a 22-gauge needle. The suspension of limbal epithelial cells was placed in supplemented media (Dulbecco's modified Eagle's medium and Ham's F12 (1:1) (Invitrogen, U.K.) including 5 % fetal bovine serum (Hyclone, Fisher UK) 2 ng/mL human epidermal growth factor, 5 mg/mL insulin and penicillin streptomycin (Life technologies, UK), and seeded onto either AM or compressed collagen gels (lining the bottom of tissue culture inserts). The upper surfaces of the collagen gels were coated with laminin (1.5 µg/cm², Invitrogen, U.K) for 2 hours prior to cell seeding. Cultures were incubated at 37 °C under 5 % CO₂ for 3–4 weeks and the cell culture medium was replaced every 2 days⁶⁵.

Immunohistochemistry

1 Limbal epithelial stem cells expanded upon AM or collagen gels were embedded in optimal
2 cutting tissue (O.C.T). compound (Jung tissue freezing medium, Germany), snap-frozen in liquid
3 nitrogen and stored at -80°C prior to being sectioned. Cryostat sections (7 µm thick) were placed
4 onto poly-lysine coated slides and air-dried for 2 hours. Slides were then fixed in 100% methanol
5 at -20°C for 15 minutes and in acetone (-20°C) for 5 minutes before being washed in PBS.
6 Tissue sections were incubated with 1% goat serum or bovine serum albumin (BSA) at room
7 temperature for 30 minutes to block non-specific binding. Blocked sections were incubated with
8 anti-cytokeratin 3 (K3) (1:50, mouse, monoclonal, Chemicon, UK) or anti-collagen IV (1:100,
9 mouse, monoclonal; Progen) primary antibodies overnight at 4°C then washed for 5 minutes in
10 PBS. Horse anti-mouse (1:50, Vector Labs, UK) fluorescent secondary antibodies were applied
11 for 60 minutes at room temperature. Unbound secondary antibodies were removed by washing in
12 PBS and sections were mounted under glass cover slips using DAPI mounting media (Vector
13 Labs, U.K.). Negative controls were performed by replacing the primary antibody with PBS.
14 Mounted sections were examined by fluorescence microscopy (Imager A1, Zeiss, Germany).
15
16 Gel electrophoresis and Western blotting
17 The isolation of limbal epithelial cells cultured on AM or compressed collagen gels was achieved
18 by incubation of the constructs in 0.05% trypsin (Sigma-Aldrich) for 5 minutes followed by the
19 use of cell scrapers. Cellular proteins were then separated by one-dimensional sodium dodecyl
20 sulphate-polyacrylamide gel electrophoresis using 10% gels. The separated proteins were
21 transferred to polyvinylidene difluoride membranes and nonspecific binding to membranes was
22 blocked by incubation with 5% BSA dissolved in 1x TBS-Tween (TBS-T) (20mM Tris-base,
23 0.14M NaCl, 0.1% Tween-20; pH 7.6). Membranes were then incubated with anti-K3 and anti-

GAPDH primary antibody (1mg/ml) diluted in 2% BSA dissolved in 1x TBS-T at 4°C overnight. Blots were washed for 45 minutes in 1x TBS-T before incubation with anti-mouse conjugated secondary antibody (1:4000 dilution) for 2 hours at room temperature. Proteins were detected on X-ray film using an enhanced chemiluminescence system (Thermo scientific, U.S.A). For analysis of collagen protein fragments following plastic compression, the fully compressed and partially compressed collagen gels we solubilized in 0.5 M acetic acid overnight. Equal amounts of collagen protein were mixed with sample buffer and loaded onto 4-15 % Tris-Glycine extended precast gels (Bio-Rad). Electrophoresis was carried out at a constant voltage of 140 V for 1 hour under non-reducing conditions. Following electrophoresis the gels were stained in 0.1 % Coomassie blue stain overnight.

Real time PCR (QPCR)

RNA extraction was performed using TRI reagent (Invitrogen) as per the standard protocol. Purified RNA from epithelial cells expanded upon collagen gels was either frozen at -80°C for up to 1 week or used immediately for cDNA synthesis. RNA purity and concentration was quantified using a Nanodrop 2000 UV spectrometer and cDNA synthesis was performed immediately after quantification. 1µg of RNA was loaded according to the standard protocol for Maxima cDNA synthesis kit (Fermentas). Synthesis was undertaken on a Techne TC-Plus thermal cycler. qPCR analysis of the resultant cDNA was undertaken with exon spanning In house primers against K3 using QuantiTect SYBR Green PCR Kit (Qiagen) on a Illumina Eco qPCR machine (n=3). All primer sets were validated with a high resolution melt and amplified fragments were run on 2% TBE gels to verify fragment size matched predicted levels.

1 Rheology

2 The mechanical properties of AM samples and compressed collagen gels were determined using
3 a controlled stress AR-2000 rheometer (TA instruments, U.K.) with plate-plate geometry.
4 Preliminary oscillatory stress sweeps were performed at a fixed frequency of 2π radians between
5 0.1-100 Pa at 10°C for AM (to prevent sample drying) or 37°C for collagen gels to determine the
6 linear viscoelastic region of our substrates. Following this, frequency sweeps were performed
7 with the machine in oscillatory mode over an angular frequency range (ω) of 0.1–100 rad/s under
8 a controlled stress of 10 Pa and 5 Pa for partial and fully compressed gels respectively and 1 Pa
9 for AM substrates. The wet weight of the AM and collagen gels before and after rheology did
10 not noticeably change.

11

12 Scanning electron microscopy and atomic force microscopy

13 Both AM and compressed collagen gel samples were fixed in 2.5% glutaraldehyde for 2 hours at
14 4°C and washed 3 times for 10 minutes with distilled water. Samples were then post-fixed in 1%
15 aqueous osmium tetroxide for 2 hours then washed with distilled water before being dehydrated
16 in graded ethanol (25%, 50%, 70%, 90% and 100%) for 15 minutes each. Samples were
17 transferred to a critical point dryer before mounting onto aluminium stubs after which samples
18 were sputter coated with gold and examined using an SEM (FEI Quanta FEG 600, UK) or AFM
19 (DualScope, DME, Denmark). AFM images for compressed collagen gels were acquired in non-
20 contact “tapping” mode over a randomly selected region of $50\mu\text{m} \times 50\mu\text{m}$. The resultant surface
21 topography data was processed using line-wise correction and tilt removal algorithms available
22 via the “Scanning Probe Image Processor” (SPIP) package. Histograms were normalised such
23 that the sum of all frequency bins was unity.

Statistical analysis

A Student's two tailed t-test was performed using Microsoft Excel to determine the statistical significance of amniotic membrane stiffness (n=3) and thickness (n=10). Results are presented as an average with a standard deviation of the mean where $p < 0.01$ was considered significant.

Results

Differences in amniotic membrane sampling and preparation influence corneal epithelial cell differentiation

Previously, corneal epithelial progenitor cells were reported to grow efficiently on amniotic membrane (AM) and eventually form a well-organized epithelial tissue, which is suitable for transplantation⁸⁻¹⁰. In this study we first confirmed that corneal epithelial stem cells are successfully cultured on differently prepared AM samples and more importantly we quantified their status of differentiation. A marker against corneal epithelial cell differentiation (K3) was probed in cells expanded upon proximal (AM samples from areas adjacent to the placental disc), distal (AM samples approximately 10cm from the placental disc), denuded AM (native AM epithelial cells were physically removed) and artificially stiffened AM (UV/riboflavin crosslinked). Immunohistochemical analysis showed very weak K3 expression in corneal epithelial cells on proximal AM (Figure 1A) whereas K3 expression was shown to increase on distal AM (Figure 1B), however K3 was most strongly expressed within cells cultured on denuded distal AM and artificially stiffened AM (Figure 1C and D). Western blotting confirmed this difference in corneal epithelial cell differentiation. K3 (64 KDa) was strongly expressed in

cells cultured on distal AM when compared to proximal AM (Figure 1E). It is known that cell differentiation can be influenced by chemical, topographical and material cues^{35-37, 39-41}. Therefore to fully identify the underlying factor within AM further studies were undertaken.

Amniotic membrane surface structure

The surface structure of proximal and distal AM was examined by scanning electron microscopy (SEM). SEM showed the native AM epithelial cells (following freeze and thawing) on the proximal AM surface were more tightly compacted (Figure 2C) than native epithelial cells on the distal AM (Figure 2A). However once denuded, the structural collagen fibres comprising AM were fully exposed (Figure 2E). As an important extracellular matrix composite protein, collagen IV was detected in all AM samples. Immunohistochemistry results showed that collagen IV was similarly localised beneath native AM epithelial cells on proximal (Figure 2B), distal (Figure 2D) and on the surface of denuded AM (Figure 2F).

Measurements of human amniotic membrane thickness

It has been previously reported that material thickness is closely correlated with mechanical stiffness if all other properties (density, organization, etc.) are held constant⁴². Proximal, distal and denuded distal AM had a mean thickness of 115.6µm (±20.7), 63.61µm (±13.0) and 9.81µm (±4.3) respectively. The proximal AM thickness was significantly greater than the distal AM and denuded AM (p<0.01), and distal AM was significantly different from denuded distal AM (p<0.01) as shown in Figure 1.

Measuring the dynamic shear moduli of natural biological substrates (AM samples)

1 Shear rheology was used to quantify the mechanical properties of differently prepared AM
2 samples (Figure 3). Here the dynamic elastic shear modulus (G') is a measure of material
3 stiffness (assuming no depth dependent differences occur in collagen density and organization
4 within the tissue). Preliminary stress sweeps were carried out to determine the linear viscoelastic
5 regime of all samples (Figure 3A) and further frequency sweep measurements allowed us to
6 characterize changes in the mechanical properties of amniotic membrane over a range of
7 frequencies (Figure 3B). Table 1 shows differences in the stiffness of amniotic membrane where
8 G' measurements from frequency sweep data were compared at 1 rad s^{-1} . Sampling distance
9 from the placenta and subsequent processing of the AM results in an increased measure of
10 mechanical stiffness. This trend is proportional to the increased K3 protein levels expressed in
11 cells on respective substrates.

12
13 Measuring the dynamic shear moduli of compressed collagen gels

14 Oscillatory shear rheology was used to compare the viscoelastic properties of fully compressed
15 and partially compressed collagen gels where substrate stiffness (G') was quantified following
16 preliminary stress sweeps (Figure 3C). Frequency sweep measurements (Figure 3D) show that
17 although both materials can exhibit a weak frequency dependent mechanical response. There was
18 a significant difference in stiffness between FC and PC collagen gels.

19
20 Compressed collagen gels surface structure

21 Scanning electron microscopy (SEM) and atomic force microscopy (AFM) analysis was
22 employed to characterize the nano-structure of our fully compressed and partially compressed
23 collagen gels (Figure 4). We found that the size and orientation of collagen fibrils on the surface

of both materials were highly comparable by SEM. It was evident that both fully compressed and partially collagen gels possessed similar surface features in terms of topographical mapping and frequency of peak heights over the length scales probed by the AFM.

Compressed collagen gels of different stiffness influence corneal epithelial cell differentiation QPCR, Western blotting and immunostaining showed significant differential K3 expression in limbal epithelial cells on collagen gels of different stiffness (Figure 5). On the less stiff partially compressed collagen gels epithelial differentiation marker K3 was weakly expressed however on stiffer fully compressed collagen gels K3 expression was markedly stronger (Figure 5A).

Laminin coating of the collagen gel was shown to be similarly distributed across each gel type (Figure 5B). Western blotting and QPCR confirmed that the overall expression of K3 in all the stratified epithelial cells was higher on the stiffer collagen gels (Figure 5C and D). There existed no obvious differences in the proportions of type I collagen fragments (gamma, beta and alpha) between the fully and partially compressed collagen gels (Figure 5E) once the formed gels had been re-solubilized and separated by gel electrophoresis. This suggests that the composition of the collagen gels was not altered by plastic compression.

Discussion

Amniotic membrane as a substrate for the ex vivo expansion of corneal stem cells has been used in the clinical treatment of ocular surface disorders for over a decade^{20, 43}. Previously we have demonstrated that the manner in which the AM is prepared, prior to its use as a substrate for corneal epithelial stem cell expansion, can strongly influence cell differentiation¹⁴. However the

mechanisms behind this were previously not understood. By building upon our work detailing the structural differences inherent to AM we are now able present novel findings, which describe a direct relationship between AM substrate stiffness and the level differentiation within a population of corneal stem cells expanded upon it.

K3 is a type II cytokeratin which has been used successfully in corneal cell biology for more than a quarter of a century^{11, 12, 14, 44} becoming universally accepted as a robust marker for terminally differentiated corneal epithelial cells across a wide range of mammalian models⁴⁵⁻⁴⁷.

This corneal epithelial cell differentiation marker showed distinct expression by immunohistochemistry, Western blotting and QPCR within epithelial cells cultured on the differently prepared collagen substrates .

Shear rheology was applied to measure the precise difference in viscoelastic properties between the collagen based substrates examined. Within AM the distal samples had a greater G' (stiffness) than the proximal samples (Figure 3). We also showed that the distal region is significantly thinner than the proximal region (Figure 1). Furthermore we provide evidence that the denuding process not only thins the AM further but also increases its mechanical stiffness (Figures 1 and 3). These results support the conclusions of Benson-Martin et al. who measured the stiffness (Young's modulus) of thick and thin AM and found that the elastic modulus decreased with increasing AM thickness⁴². A possible explanation as to why the distal (thinner) AM is stiffer than the proximal (thicker) AM is a difference in the alignment of its constituent collagen fibres that make up the bulk of the materials structure. Previously we have shown, by synchrotron small angle x-ray scatter, that distal AM contains a higher degree of anisotropy within its

1 collagen fiber arrangement than does the proximal AM³³. Anisotropy within fibrillar materials is
2 known to substantially increase its material stiffness⁴⁸.
3
4 Expanded corneal epithelial cells have previously been shown to exhibit a distinct variation in
5 their level of differentiation in response to different culture conditions i.e. in the presence (intact
6 AM) or absence (denuded AM) of native amniotic epithelia. Differentiation was observed to
7 markedly increase in corneal epithelial cells expanded upon denuded AM¹⁴. In the present study
8 the rheology results show that denuding the AM increases its stiffness (Figure 3 and Table 1) and
9 that this difference in the substrates mechanical property, may explain the previously observed
10 variation in limbal cell differentiation on intact and denuded AM¹⁴. As shown in Figure 1 the
11 denuding process dramatically reduces the material's thickness, possibly by dehydration via
12 stretching or compression following vigorous use of a hand held cell-scraping tool. In support of
13 this theory we and others have previously shown that dehydration of a similar biomaterial (a type
14 I collagen gel) can significantly increase its mechanical stiffness^{49 50}.
15 Artificially stiffening AM using UV light in the presence of riboflavin was employed as a
16 positive control for the effect of substrate stiffness on limbal epithelial cell differentiation.
17 Photochemical cross-linking is a rapid and efficient process with little toxicity⁵¹. Riboflavin acts
18 as a photomediator, creating free radicals, in the presence of ultraviolet A (UVA) light to induce
19 new chemical bonds bridging amino groups of collagen fibrils⁵² and subsequently increase
20 mechanical stiffness. We have previously applied this technique to compressed collagen gels and
21 shown that UVA/riboflavin treatment has no effect on epithelial growth or surface structure⁵³.
22 This approach has also been successfully used to stiffen human corneas in the treatment of
23 keratoconus⁵⁴ a disease which affects the mechanical properties of the cornea.

Besides the influence that a substrate's mechanical properties can have on cell behavior, it is also known that cells respond to chemical and topographical cues³⁹⁻⁴¹. Chemical cues including soluble proteins, growth factors, cytokines as well as extracellular matrix ligand-bound receptor molecules are all able to effect cell differentiation⁵⁵. Cells cultivated on scaffolds with well-defined topographical features influence focal adhesion complexes and direct cell differentiation or proliferation⁵⁶. Therefore the influence of these cues on the levels of differentiation in expanded limbal stem cells also needed to be addressed.

Fresh AM is known to be enriched with various growth factors, anti-angiogenic and anti-inflammatory proteins^{30,31} and these soluble fractions have previously been proposed to influence limbal stem cell differentiation^{57,58}. However more recently it has been shown that due to the necessary vigorous washing and processing of AM, prior to its use as a limbal stem cell substrate, there remains no detectable soluble fraction following one day in culture^{32,59}.

AM basement membrane integrity has also been proposed to play a key role in maintaining the expanded limbal stem cells in an undifferentiated state^{14, 17, 60}. However within our experiments we still were able to observe an increase in K3 expression with correspondingly increased levels of stiffness between proximal, intact and denuded AM despite having shown a similar basement membrane composition (collagen IV) i.e. in this model the increased K3 expression was independent of basement membrane protein expression patterns (Figure 2).

To highlight the importance of substrate stiffness in this system we replaced AM with a

1 compressed collagen gel, having shown previously that they share similar structural properties⁶¹,
2 ⁶². These compressed collagen gels were prepared entirely from collagen type I, this being the
3 predominant structural collagen in normal AM. The significance of this to the current study is
4 that we can produce fibrillar collagen gel substrates with a tractable stiffness whilst retaining a
5 similar surface topography and surface chemistry (Figures 3, 4 and 5). This allows us to
6 accurately model the effect of AM stiffness on corneal epithelial differentiation, thus providing
7 confirmation that increased material stiffness drives differentiation of limbal stem cells.

8
9 Furthermore this work also brings clearly into focus the urgent need to consider substrate
10 stiffness when designing future scaffolds for ocular surface restoration or corneal tissue
11 engineering as shown by our use of compressed collagen type I gels of tractable stiffness.

12
13
14 **Conclusions**

15 In conclusion our results demonstrate that the known differences in AM structure between
16 samples collected from anatomically distinct areas distal and proximal to the placenta can
17 influence the materials elastic modulus. Furthermore the measured differences in collagen
18 substrate stiffness (AM and compressed collagen gels) has a marked and proportional effect on
19 level of differentiation within an expanded population of limbal stem cells. This work offers an
20 elegant explanation of the previous inconsistencies observed when AM has been used both
21 clinically and experimentally. Previously no consideration was given to origin of the AM
22 (relative to the placenta) when used as a substrate for the ex vivo expansion of limbal stem cells
23 prior to transplantation. We have now shown that, when used as a substrate for corneal epithelial

stem cells, this would have a marked effect on the level of differentiation. This is particularly pertinent as the hypothesis underlying the clinical use of AM for the treatment of ocular surface stem cell disorders is that the transplanted cells should remain in a relatively undifferentiated state to maximize their clinical effect. Our work suggests that this could be optimized by use of AM collected from an area proximal to the placenta i.e. a less stiff region or by normalizing the substrates mechanical properties, chemically, prior to use.

Figure legends

Figure 1

K3 expression (red) in limbal epithelial cells cultured on (A) Proximal (B) Distal (C) Denuded and (D) UV cross-linked amniotic membrane. Cell nuclei stained by DAPI (blue). K3 expression is seen to increase its association with deeper layers of stratified cells from proximal>distal>denuded>UV treated amniotic membrane. This increase was also apparent by Western blotting against K3 with GAPDH as a loading control (E). The thickness of amniotic membrane was measured ($n \geq 9$) and a significant difference in thickness was found between distal, proximal and denuded AM (* = $P < 0.01$). Error bars equal standard deviation of the mean (F). A representative image showing the thickness measurement of distal amniotic membrane (G)

Figure 2

The ultrastructure and collagen IV expression (red) upon the surface of distal (A,B), proximal (C, D) and denuded (E, F) amniotic membrane prior to its use in the expansion of limbal epithelial cells was examined by scanning electron microscopy and immunohistochemistry respectively. Amniotic epithelial cells appear more loosely attached in the distal tissue (A). The denuding process whilst removing the amniotic epithelia does retain a similar expression pattern of collagen IV across its upper surface (F).

Figure 3

Rheology data showing the mechanical properties of proximal, distal, denuded and UV treated human amniotic membrane (A,B) and fully [FC] and partially compressed [PC] collagen gels (C,D). Oscillatory stress sweeps (A,C) and frequency sweeps (B,D) are shown for each substrate. Closed symbols refer to G' (dynamic elastic shear modulus), open symbols refer to G'' (dynamic viscous shear modulus).

Figure 4

Scanning electron micrographs from the surface of fully compressed [FC] and partially compressed [PC] collagen gels reveal a similar ultrastructure in terms of collagen fibre density and diameter (A). Atomic force microscopy across $50\mu\text{m} \times 50\mu\text{m}$ surface regions of the fully and partially compressed collagen gels revealed a similar surface topography by both 3D mapping (B) and a distribution of peak heights histogram (C).

Figure 5

Immunohistochemical staining of limbal epithelial cells on fully compressed [FC] and partially compressed collagen [PC] gels (A). K3 (red) is predominately associated with superficial cells expanded upon the stiffer fully compressed collagen gel. Nuclei counterstained with DAPI (blue). Laminin distribution (green) was similar between the fully compressed and partially compressed collagen gels, negligible amounts were detected in the control gel without laminin coating (B). Western blotting results show an increased K3 (64 kDa) expression from epithelial cells expanded upon stiff collagen gels [FC] when compared to the less stiff [PC] collagen gels (C). This difference was also quantified by QPCR showing a significant increase in K3 mRNA levels when expanded upon the stiffer collagen gels (D). Coomassie blue staining of collagen fragments in fully compressed [FC] and partially [PC] collagen gels revealed no obvious difference in the relative amounts of type I collagen fragments (gamma, beta and alpha) (D). Control is an uncompressed collagen gel.

Table 1

Dynamic elastic shear modulus measurements (G') of amniotic membrane revealed an increase in stiffness (UV/riboflavin>denuded>distal>proximal) with increasing sampling distance from the placenta and subsequent processing (removal of native epithelia and chemical cross-linking).

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